Effects of P_{SAG12}-*IPT* Gene Expression on Development and Senescence in Transgenic Lettuce[1]

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An *ipt* gene under control of the senescence-specific SAG12 promoter from Arabidopsis (P_{SAG12}-*IPT*) significantly delayed developmental and postharvest leaf senescence in mature heads of transgenic lettuce (*Lactuca sativa* L. cv Evola) homozygous for the transgene. Apart from retardation of leaf senescence, mature, 60-d-old plants exhibited normal morphology with no significant differences in head diameter or fresh weight of leaves and roots. Induction of senescence by nitrogen starvation rapidly reduced total nitrogen, nitrate, and growth of transgenic and azygous (control) plants, but chlorophyll was retained in the lower (outer) leaves of transgenic plants. Harvested P_{SAG12}-*IPT* heads also retained chlorophyll in their lower leaves. During later development (bolting and preflowering) of transgenic plants, the decrease in chlorophyll, total protein, and Rubisco content in leaves was abolished, resulting in a uniform distribution of these components throughout the plants. Homozygous P_{SAG12}-*IPT* lettuce plants showed a slight delay in bolting (4–6 d), a severe delay in flowering (4–8 weeks), and premature senescence of their upper leaves. These changes correlated with significantly elevated concentrations of cytokinin and hexoses in the upper leaves of transgenic plants during later stages of development, implicating a relationship between cytokinin and hexose concentrations in senescence.

Leaf senescence is a type of programmed cell death characterized by loss of chlorophyll, lipids, total protein, and RNA (Smart, 1994; Gan and Amasino, 1997). This process is believed to be an evolutionarily acquired, active genetic trait that contributes to plant fitness, for example, by remobilizing nutrients from vegetative tissues to reproductive organs (Oh et al., 1997). The biological importance and potential for improvement of crop characteristics, particularly plant productivity and postharvest storage, have prompted extensive physiological, molecular, and genetic analyses of leaf senescence (Nam, 1997). Senescence can be induced by environmental stress, such as low light intensity, nutrient deficiency, pathogen attack, drought, waterlogging, and detachment from the plant. Endogenous factors, including leaf age and reproductive development, also trigger senescence (Smart, 1994; Gan and Amasino, 1997).

Plant growth regulators, including auxins, gibberellins, ethylene, abscisic acid, and cytokinins, are believed to play major roles in regulating senescence. Attention has focused on cytokinins that are key components of plant senescence (Van Staden et al., 1988; Singh et al., 1992; Gan and Amasino, 1996, 1997; Buchanan-Wollaston, 1997; Nam, 1997). These compounds have been implicated in several aspects of plant development and are thought to be synthesized mainly in the roots and transported to the shoots via the xylem (Gan and Amasino, 1996). Three main approaches have been used to study the effect of cytokinins in plant senescence, namely exogenous application of cytokinin solutions, measurement of endogenous cytokinins during senescence, and transgene-encoded cytokinin biosynthesis.

Measurements show that the concentrations of endogenous cytokinins decline in plant tissues as senescence progresses (Van Staden et al., 1988). The cytokinin content of the xylem sap of sunflower (*Helianthus annuus*) and soybean (*Glycine max*) also decreases rapidly with the onset of senescence, which suggests that reduction in cytokinin transport from roots to shoots allows senescence to progress (Skene, 1975; Nooden et al., 1990). Exogenous application of cytokinins retards senescence of detached leaves (Richmond and Lang, 1957), although cytokinins are often less effective in attached tissues (Gan and Amasino, 1996). External application of cytokinins, such as dihydrozeatin and benzyladenine, has been exploited commercially to extend the shelf life of

freshly harvested vegetables and cut flowers (Kays, 1991).

Transgene-encoded cytokinin biosynthesis was initially studied in tobacco (*Nicotiana tabacum* cv Wisconsin 38) using constitutive or inducible overexpression of the *ipt* gene, which encodes isopentenyl phosphotransferase, from *Agrobacterium tumefaciens*. This enzyme catalyzes the rate-limiting step for de novo cytokinin biosynthesis (McGaw and Burch, 1995), i.e. the addition of isopentenyl pyrophosphate to the N^6 of 5'-AMP to form isopentenyl AMP (Chen, 1997). In plants, this reaction is normally catalyzed by iptase, but this enzyme is highly labile and has not been purified. Isopentenyl AMP is the precursor of all other cytokinins, of which the three most commonly detected and physiologically active forms in plants are isopentenyl adenine (IPA), zeatin (Z), and dihydrozeatin (DHZ; Mok and Mok, 1994). In earlier work, overexpression of the *ipt* gene in transgenic plants led to elevated foliar cytokinin concentrations and delayed leaf senescence, but the high cytokinin levels were largely detrimental to growth and fertility (Medford et al., 1989; Smart et al., 1991; Li et al., 1992; Wang et al., 1997a, 1997b). To circumvent these effects, Gan and Amasino (1995) devised a strategy, based on autoregulated cytokinin production, which delayed leaf senescence in transgenic tobacco without altering plant phenotype. This strategy exploited a highly senescence-specific promoter (P_{SAG12}) from an Arabidopsis gene encoding a Cys proteinase (Lohman et al., 1994), fused to the *ipt* gene (synonym *tmr* gene) from the Ti plasmid of *A. tumefaciens* (Hidekamp et al., 1983). The P_{SAG12}-*IPT* gene was reported to be activated only at the onset of senescence in the lower mature leaves of tobacco. This resulted in cytokinin biosynthesis in the leaves, which inhibited leaf senescence and, consequently, attenuated activity of the P_{SAG12}-*IPT* gene, preventing cytokinin overproduction.

The ability to delay leaf senscence has potential for crop improvement. However, to date, the effect of *ipt* expression in transgenic plants has been assessed mainly in a limited number of Solanaceous spp. (Gan and Amasino, 1996), although there are brief reports of the introduction of P_{SAG12}-*IPT* into rice (*Oryza sativa*; Fu et al., 1998), cauliflower (*Brassica oleracea*; Nguyen et al., 1998), and lettuce (*Lactuca sativa* L. cv Evola; McCabe et al., 1998). Leaf senescence is a problem in vegetables such as lettuce, with leaf yellowing and wilting determining their relatively short postharvest storage life. To date, studies of the retardation of lettuce leaf senescence have relied on external application of plant growth regulators. For example, application of dilute solutions of ascorbic acid, EDTA, gibberellic acid, and 2,4-dichlorophenoxyacetic acid extended the shelf life of shredded lettuce by up to 300% (Rossit and DeQuoy, 1982), whereas Aharoni (1989) demonstrated that lettuce leaf discs incubated on solutions of gibberellic acid,

kinetin, or indole acetic acid exhibited retardation of chlorophyll degradation.

This paper reports the effects of P_{SAG12}-*IPT* expression on leaf senescence, plant morphology, and assimilate partitioning in transgenic lettuce. Differences between the effects of expression of P_{SAG12}-*IPT* in lettuce and tobacco are also addressed. In addition, the potential commercial applications (increased shelf life, fungus resistance, and reduced nitrate content) of transgene-encoded autoregulated cytokinin biosynthesis in this crop are discussed.

RESULTS

Transgenic Plant Production

Four T_0 lines (1394.1, 1394.53, 1394.75, and 1394.79), which showed cosegregation of the delayed senescence phenotype with reporter gene expression in their seed progeny (T_1 plants), were selected for further analysis and the production of homozygous T_2 lines (1394.1.7, 1394.53.1, 1394.75.5, and 1394.79.9). Four azygous lines (1394.1.4, 1394.53.6, 1394.75.6, and 1394.79.5) were selected from T_1 plants, and maintained as controls for their respective homozygous counterparts.

Southern Analysis

To confirm that the chosen lines were independent transgenic lines, T-DNA/plant DNA junction fragment analysis of the right side of the T-DNA was performed on genomic DNA from 24 β-glucuronidase (GUS)-positive T_0 plants derived from 16 independently inoculated explants. Hybridization patterns with the *ipt* probe confirmed T-DNA integration into the lettuce genome and allowed the identification of 11 independently transformed lines, each with a single P_{SAG12}-*IPT* gene insert.

Leaf Senescence and Morphology

The first phenotypic trait assessed was delayed senescence of lower leaves. Delayed leaf senescence was not always obvious in T_0 P_{SAG12}-*IPT* lettuce plants because the latter were at different stages of growth due to variation in the time of shoot regeneration from inoculated explants. However, delayed leaf senescence was clearly visible in T_1 plants and cosegregated with GUS expression, although GUS-positive T_1 plants from the same parent exhibited variation in the extent of the delayed senescence phenotype. Following self-pollination of the T_1 plants, segregation analysis of GUS expression in the resulting T_2 seedlings revealed that the T_1 plants with pronounced delayed senescence were homozygous for the T-DNA; those with less pronounced delayed senescence were hemizygotes, indicating a gene dosage effect. Detailed analysis of delayed senescence was performed using plants from the homozygous T_2

line 1394.1.7, and the results were verified by analyzing the three remaining T_2 homozygous lines, 1394.53.1, 1394.75.5, and 1394.79.9.

As in the T_1 generation, the first sign of delayed leaf senescence in homozygous T_2 plants (1394.1.7) was a difference in the chlorophyll content of cotyledons of GUS-positive P_{SAG12}-*IPT* plants compared with azygous controls (1394.1.4) at 37 to 39 d postsowing (dps; 15.2 ± 1.5 and 2.1 ± 1.8 μg cotyledon^{-1}, respectively; $n = 20$). At 42 to 45 dps, the chlorophyll content of the primary true leaves of transgenic plants (1394.1.7) was also significantly greater than in azygous plants (1394.1.4; 11.0 ± 1.0 and 3.0 ± 1.0 μg cm^{-1}, respectively; $n = 10$; Fig. 1, a and b). Following cutting of heads at 60 dps and storage for 7 d, the outer leaves of heads of the four azygous lines were yellow and necrotic after this storage period, whereas leaves of the four homozygous lines of T_2 P_{SAG12}-*IPT* heads retained their chlorophyll (Fig. 1, c and d). In intact plants, the basal leaves of trangenic plants did not senesce in the same way as those of azygous plants. Instead of losing both chlorophyll and turgor as in azygous (1394.1.4) plants, the basal leaves of transgenic plants (1394.1.7) lost turgor, but remained green for approximately 7 d longer than those of azygous plants, before becoming necrotic. This delayed senescence was also expressed in the three homozygous lines 1394.53.1, 1394.75.5, and 1394.79.9, with none of the basal leaves of the transgenic plants ($n = 40$ of each line) showing signs of senescence at 60 dps (Fig. 1e). In contrast, all their respective azygous controls ($n = 40$ of each line) exhibited senescent primary leaves (Fig. 1f). The number of senescent leaves was significantly less in T_2 transformed plants of the line 1394.1.7 compared with azygous control plants (line 1394.1.4) over 49 to 81 dps (Fig. 2).

Other phenotypic differences were also observed, but these only became evident in homozygous T_2 populations. First, the leaves of homozygous T_2 P_{SAG12}-*IPT* (1394.1.7) seedlings were significantly smaller than the leaves of wild-type and azygous (1394.1.4) controls (0.8 ± 0.3 cm^2 and 3.3 ± 2.7 cm^2, respectively) at 28 dps. However, by 60 dps there were no differences in head radius, height, or fresh weight between transgenic and azygous plants. Second, T_2 P_{SAG12}-*IPT* plants of lines 1394.1.7, 1394.53.1, 1394.75.5, and 1394.79.9 showed a slight delay (approx. 6 d) in bolting, flower bud formation, and panicle development. The stems and panicle branches of these four homozygous P_{SAG12}-*IPT* plant lines were thicker and less green than those of azygous plants (Fig. 1g). Transgenic plants required 4 to 8 weeks longer to produce flowers and to set seed. The panicles remained green for up to 4 months longer in homozygous P_{SAG12}-*IPT* (1394.1.7) plants than in their azygous controls (1394.1.4).

Quantification of total chlorophyll and protein in leaves from different positions on the stems of P_{SAG12}-*IPT* lettuce and azygous plants at 30, 60, and 96 dps revealed that there was no difference between transgenic and azygous plants at 30 dps. However, at 60 dps when plants had formed heads and senescence was visible in the lower leaves of azygous plants, chlorophyll and protein distribution was significantly different between T_2 plants of the four transgenic lines and azygous plants. Compared with their azygous controls, total chlorophyll content in the lower leaves was up to 3-fold higher in homozygous P_{SAG12}-*IPT* plants of lines 1394.1.7, 1394.53.1, and 1394.75.5 and up to 9-fold higher in 1394.79.9 homozygous P_{SAG12}-*IPT* plants. Protein content in the lower leaves was up to 2-fold higher in 1394.1.7, 1394.53.1, and 1394.75.5 homozygous P_{SAG12}-*IPT* plants and 6-fold higher in 1394.79.9 homozygous P_{SAG12}-*IPT* plants. This difference was even more pronounced at 96 dps when the plants had bolted (Fig. 3). At 96 dps, transgenic plants of line 1394.1.7 exhibited a uniform distribution of chlorophyll, LSU Rubisco, and protein from the basal to the upper leaves, whereas azygous plants (line 1394.1.4) showed a gradient of chlorophyll, LSU Rubisco, and protein (Fig. 4) from the upper leaves to the basal leaves, with the upper leaves exhibiting greatest concentrations. However, the leaf total chlorophyll, LSU Rubisco, and protein in entire transgenic 1394.1.7 plants at 96 dps were lower relative to the values for azygous 1394.1.4 plants by $32\% \pm 3.4\%$, $10\% \pm 1.85\%$, and $32\% \pm 2.3\%$, respectively. As a consequence, compared with the upper leaves of control plants, those of transgenic plants were a uniform, yet paler green. Azygous plants also had senescent basal leaves, indicating a redistribution of metabolites. The upper leaves of transgenic plants exhibited premature senescence just prior to flowering (data not shown).

Sugar Distribution

Analysis of foliar sugar levels revealed a significant difference in hexose distribution between P_{SAG12}-*IPT* 1394.1.7 plants and azygous 1394.1.4 plants at different developmental stages (Fig. 5). In both groups of plants, the concentration of Suc in the upper leaves decreased during head formation (60 dps) and bolting (96 dps), indicating an overall decline in photosynthesis. A 3- to 4-fold increase in Suc occurred after the onset of flowering (between 96–123 dps), possibly resulting from starch breakdown or reduction in Suc consumption. Almost the reverse of this pattern of distribution was observed in the upper leaves for Glc and Fru. In the upper leaves of azygous plants, there was an approximately 50% increase in Fru between 36 and 60 dps followed by a 60% to 70% decline between 60 and 123 dps. The increase and decline of Glc in the upper leaves of azygous plants over 36, 60, 96, and 123 dps followed approximately the same pattern as Fru, but was much less pronounced (Fig. 5). In P_{SAG12}-*IPT* plants, the concentrations of hexoses in the upper and middle leaves continued to increase
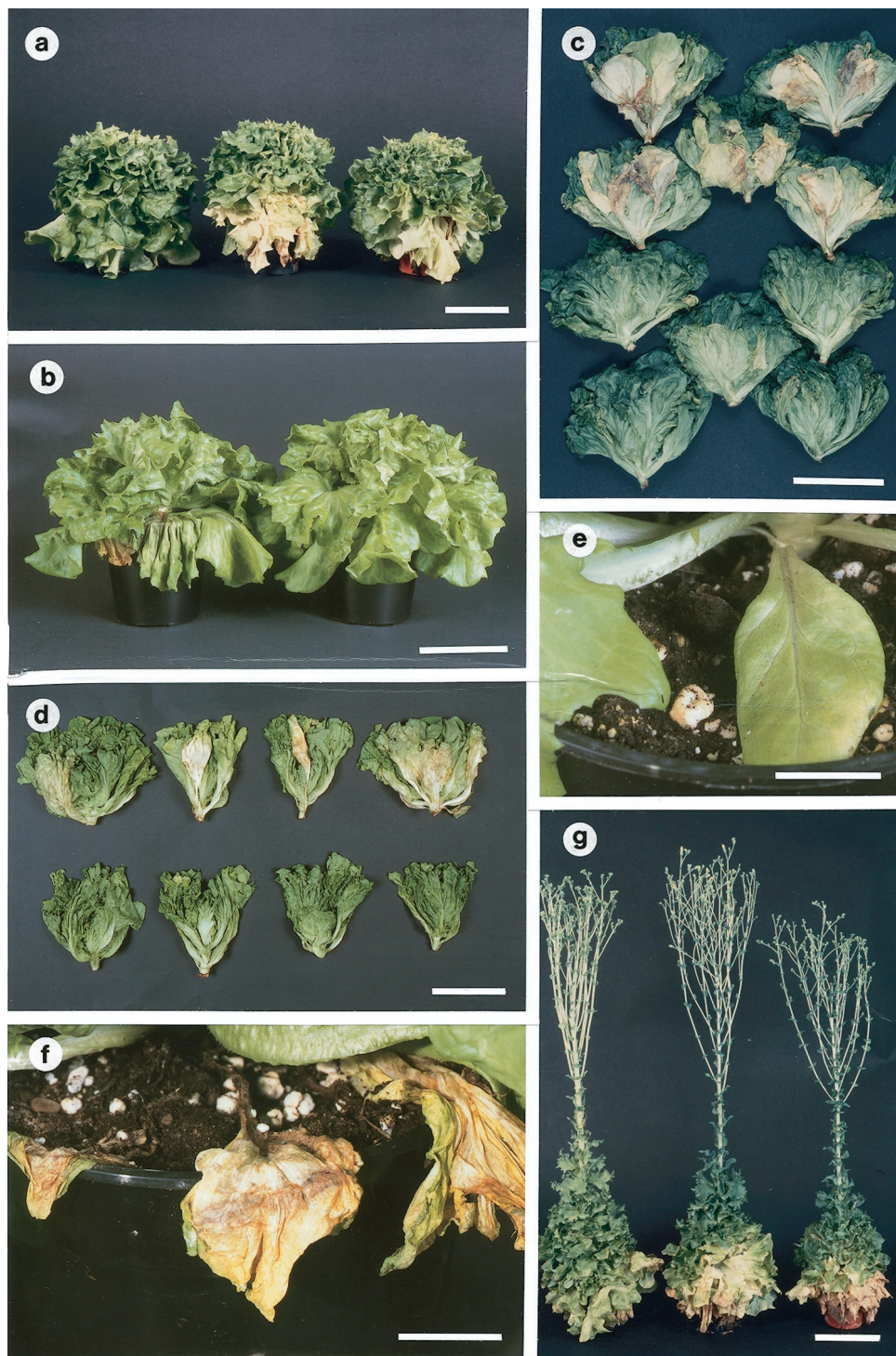
Figure 1. a, T_2 P_{SAG12}-*IPT* plant of line 1394.1.7 (left), compared with an azygous (1394.1.4) plant (center) and a wild-type plant (right) at 60 dps. b, T_2 P_{SAG12}-*IPT* (1394.79.9) plant (right), compared with an azygous (1394.79.5) plant (left) at 60 dps. c and d, Heads of azygous (1394.1.4 and 1394.79.5) plants (top) and P_{SAG12}-*IPT* (1394.1.7 and 1394.79.9) plants (bottom), cut at 60 dps and stored for 7 d, showing chorophyll retention in leaves of the transgenic plants. e, Basal leaf of a P_{SAG12}-*IPT* (1394.79.9) plant at 60 dps, with no evidence of senescence. f, Senescing, basal leaf of an azygous (1394.79.5) plant at 60 dps. g, Transgenic (1394.1.7), azygous (1394.1.4), and wild-type plants (left to right) at 96 dps. The transgenic plant has thicker panicles, less green upper leaves, but less senescence of the lower leaves compared with the azygous and wild-type plants. Bars = 10 cm (a–d and g) and 2.5 cm (e and f).
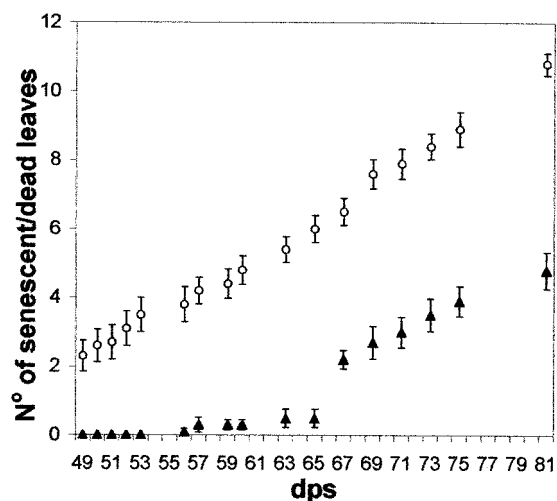
Figure 2. Progression of leaf senescence determined by counting the number of yellow/brown leaves of homozygous (1394.1.7) P$_{SAG12}$-*IPT* (▲) and corresponding azygous (○) plants from 49 to 81 dps (n = 10). Error bars represent SE.

from 36 to 96 dps when the concentrations were 3- to 4-fold higher than in controls. Glc and Fru concentrations subsequently declined in transgenic plants between 96 and 123 dps (Fig. 5). Hexoses, particularly Fru, also increased in the stems of transgenic plants, this increase being accompanied by elevated stem water content (Fig. 6). Overall, although hexoses followed an expected pattern of increase and decline during development of P$_{SAG12}$-*IPT* 1394.1.7 plants compared with azygous controls, the increase of these sugars appeared to be amplified. The foliar sugar content and distribution in the leaves of P$_{SAG12}$-*IPT* 1394.1.7 plants, as well as in the leaves of plants from the three additional homozygous P$_{SAG12}$-*IPT* lines 1394.53.1, 1394.75.5, and 1394.79.9, were examined at 70 dps. Glc and Fru levels in the upper leaves of transgenic plants were up to 2-fold higher than in azygous plants, but differences were not observed in Suc concentrations.

Cytokinin Analysis

At 60 dps, no significant difference was detected in cytokinin distribution between transgenic (1394.1.7) and azygous (1394.1.4) plants despite the "stay-green" phenotype of the transgenic plants. However, compared with azygous plants, transgenic flowering plants (96 dps) showed a large increase in isopentenyl adenine riboside (IPAR), zeatin riboside (ZR), and dihydrozeatin riboside in their upper parts (Fig. 7), with a predominance of IPAR and ZR. Free-base DHZ and DHZ nucleosides were detected at much lower concentrations than IPA, Z, and their derivative nucleoside forms.

Effects of Nitrogen Limitation

Results are presented of two experiments conducted on separate occasions. The first experiment

(Fig. 8) compared the homozygous T$_2$ P$_{SAG12}$-*IPT* line 1394.1.2 with wild-type plants; azygous plants were used as controls in the second experiment. In the latter, a homozygous T$_2$ P$_{SAG12}$-*IPT* line 1394.1.7 was compared with its azygous segregant (1394.1.4), both of which were derived from the same T$_0$ parent as line 1394.1.2. Both experiments yielded similar results. Apart from a slight reduction in total nitrate content of entire transgenic plants, significant differences were not observed between P$_{SAG12}$-*IPT* and
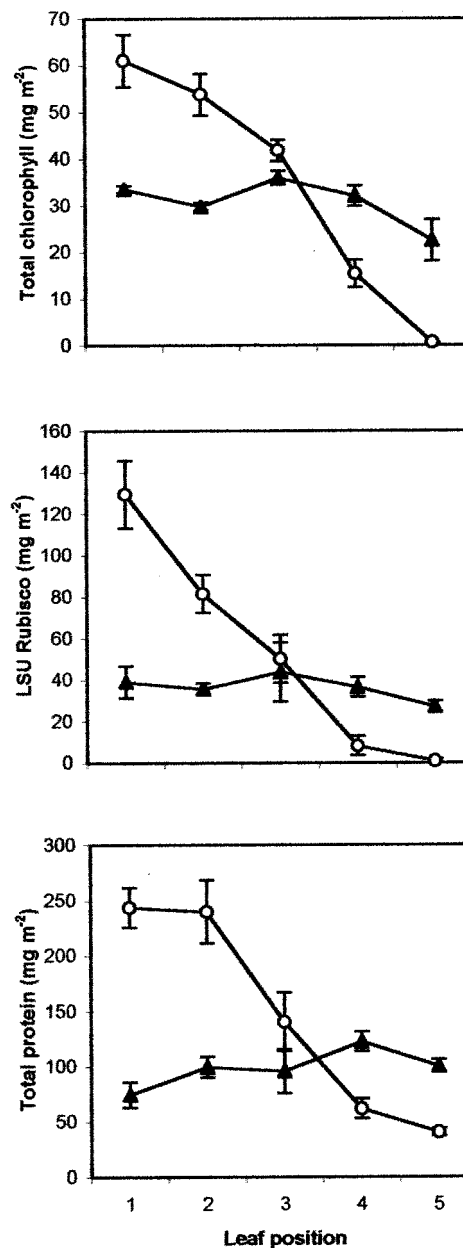

Figure 3. Distribution of chlorophyll, large subunit (LSU) Rubisco, and soluble protein in leaves of homozygous bolted (1394.1.7) P$_{SAG12}$-*IPT* (▲) and corresponding azygous (○) plants of approximately the same height at 96 dps. Leaves were taken every 20 cm from the top (position 1) of the plants to the base of the stems (position 5; n = 3). Error bars represent SE.

Figure 4. SDS PAGE analysis of LSU and small subunit Rubisco in leaves from homozygous P_{SAG12}-*IPT* plants (lanes 1 [top leaf]–5 [bottom leaf]) and azygous plants (lanes 6 [top leaf]–10 [bottom leaf]).
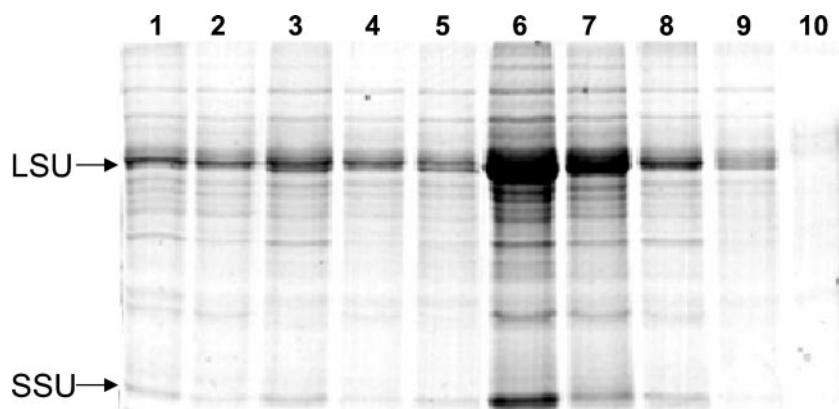
control plants grown in conditions where nitrogen was not limiting. Entire P_{SAG12}-*IPT* and control plants grown in the absence of nitrogen exhibited a reduction in total nitrate, total nitrogen, and growth rate (measured as fresh weight), compared with plants grown when nitrogen was not limiting. However, although the lower leaves of control (wild-type) plants became yellow, the lower leaves of P_{SAG12}-*IPT* plants retained their chlorophyll and did not show

signs of senescence (Fig. 9). The effects of postharvest storage were significantly different for control (azygous and wild-type) and transgenic plants. The basal leaves of control plants grown in non-limiting conditions became yellow after 7 d of postharvest storage, whereas comparable leaves of P_{SAG12}-*IPT* plants retained their chlorophyll. In plants that had been grown in nitrogen-limiting conditions, yellowing increased in the basal leaves of controls after 7 d post-
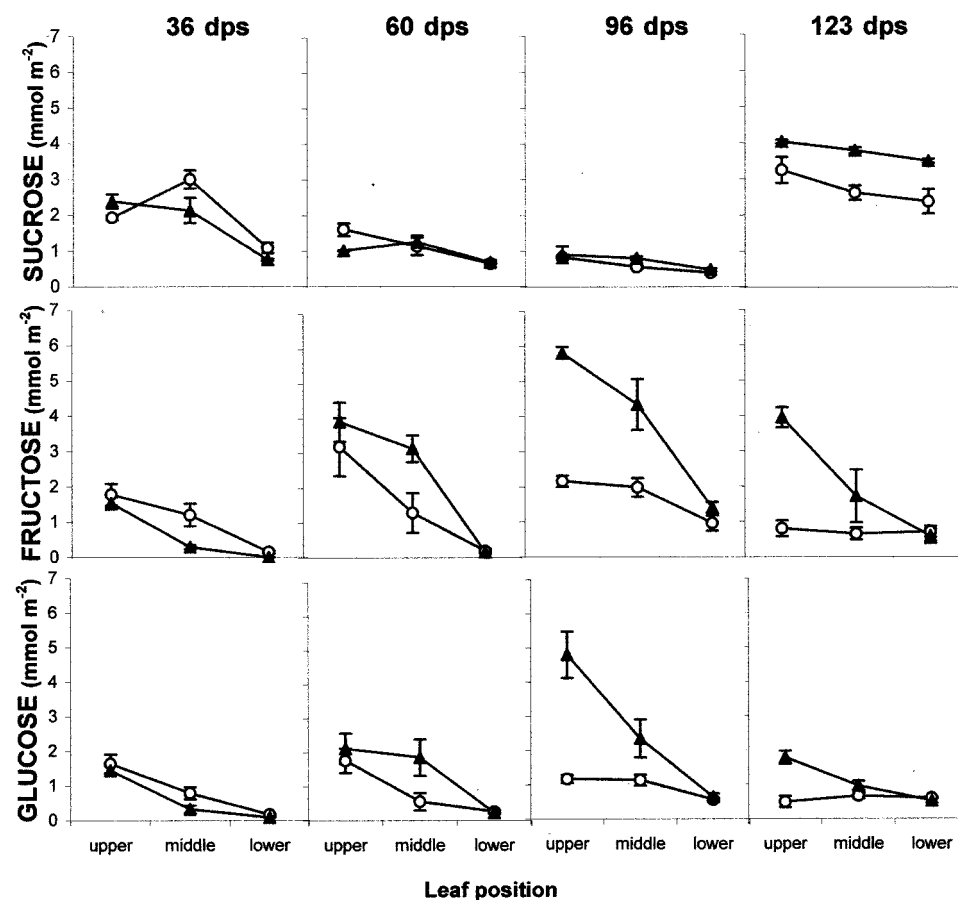

Figure 5. Distribution of sugars in upper (immediately below panicle), middle, and lower leaves of homozygous (1394.1.7) P_{SAG12}-*IPT* (▲) and corresponding azygous (○) plants 36 dps (plants at five–six leaves stage), 60 dps (mature heads), 96 dps (bolted/preflowering), and 123 dps (post-flowering; $n = 3$). Error bars represent SE.
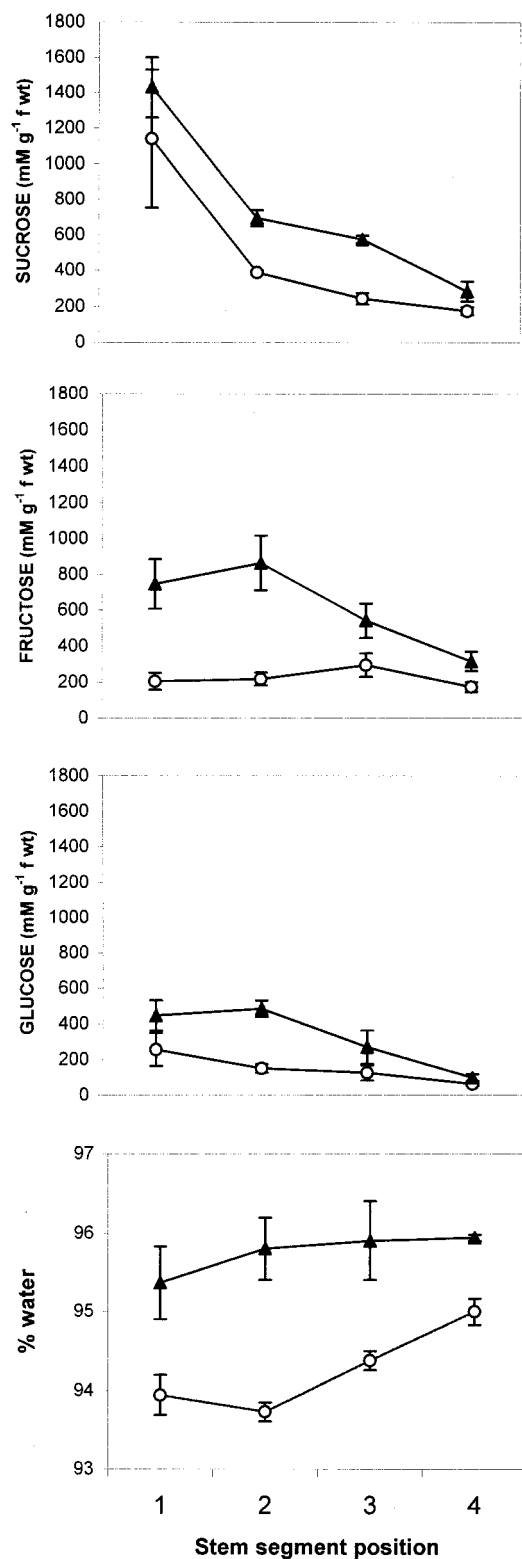
Figure 6. Distribution of sugars in upper (1; immediately below panicle), upper middle (2), lower middle (3), and bottom (4) 10-cm stem segments of P_{SAG12}-*IPT* homozygous (1394.1.7; ▲) and corresponding azygous (○) plants at 123 dps (*n* = 3). The water content is also shown of upper, upper middle, lower middle, and bottom 10-cm stem segments of homozygous P_{SAG12}-*IPT* (▲) and azygous (○) plants at 96 dps (*n* = 3). Error bars represent SE.

harvest storage, whereas leaves remained green in P_{SAG12}-*IPT* plants.

DISCUSSION

There are a number of potential applications of delayed senescence in P_{SAG12}-*IPT* lettuce. Because leaves retain their chlorophyll longer after harvesting, the most obvious application is increased postharvest quality. It is interesting that these plants also showed a significant reduction in susceptibility to infection by *Botrytis cinerea* (W. Jordi, unpublished data) because this pathogen normally infects senescent tissue. The results of nitrogen limitation showed that P_{SAG12}-*IPT* plants remained green despite loss of nitrogen and nitrates. This could provide a strategy for lowering nitrate content in lettuce cultivated on nutrient films. Limits on the nitrate content of lettuce, particularly in northern Europe, dictate that low nitrate content is an important breeding objective for this crop (Gunes et al., 1994). Removal of nitrogen from the growth medium 5 or 10 d before harvest of P_{SAG12}-*IPT* lettuce could result in up to 70% reduction in nitrate content with only a slight reduction in growth and no leaf yellowing. These adverse effects of P_{SAG12}-*IPT* on reproductive development need to be addressed if transgenic plants are to be introduced into breeding programs.

Initial activation of P_{SAG12}-*IPT* appeared to be senescence specific because, apart from the delay in senescence of the lower leaves, there was little difference in morphology, distribution of cytokinin, sugars, proteins, and chlorophyll in young plants. However, seedlings of P_{SAG12}-*IPT* lettuce were initially smaller than control seedlings, a feature also observed with P_{SAG12}-*IPT*-transformed seedlings of tobacco by some of the present authors (W. Jordi, unpublished data). This might be due to activation of the transgene during the early stages of plant development. It is interesting that seeds of homozygous P_{SAG12}-*IPT* tobacco germinated approximately 2 d later than seeds of wild-type plants (W. Jordi, unpublished data).

The marked difference in phenotype of lettuce plants at bolting indicated that, for some reason, P_{SAG12}-*IPT* was not fully autoregulated during the later stages of development. Senescence would normally cause the SAG12 promoter to be expressed, resulting in the overexpression of cytokinin (Gan and Amasino, 1995). If cytokinin can attenuate senescence, then there would be a negative feedback, causing the SAG12 promoter to terminate *ipt* gene expression. In the upper leaves of transgenic plants, it appears that senescence is initiated but that the increase in cytokinin due to SAG12 activation cannot attenuate senescence and, therefore, the autoregulatory loop cannot function. This was confirmed by the large increase in IPAR and ZR in the upper parts of transgenic plants. Recent studies have shown that

Figure 7. Cytokinin content of top, upper middle, lower middle, and bottom leaves (1, 2, 3, and 4, respectively) of homozygous P_{SAG12}-*IPT* (▲) and azygous (○) plants at 96 dps (*n* = 3). Error bars represent SEM. Each data point represents cytokinins extracted from approx. 5 g f weight leaf material. Freebase forms are IPA, Z, and DHZ; nucleoside (riboside) forms are IPAR, ZR, and dihydrozeatin riboside; nucleoside (glucoside) forms are IPA-N9-GLUC, Z-N9-GLUC, and DHZ-N9-Z.
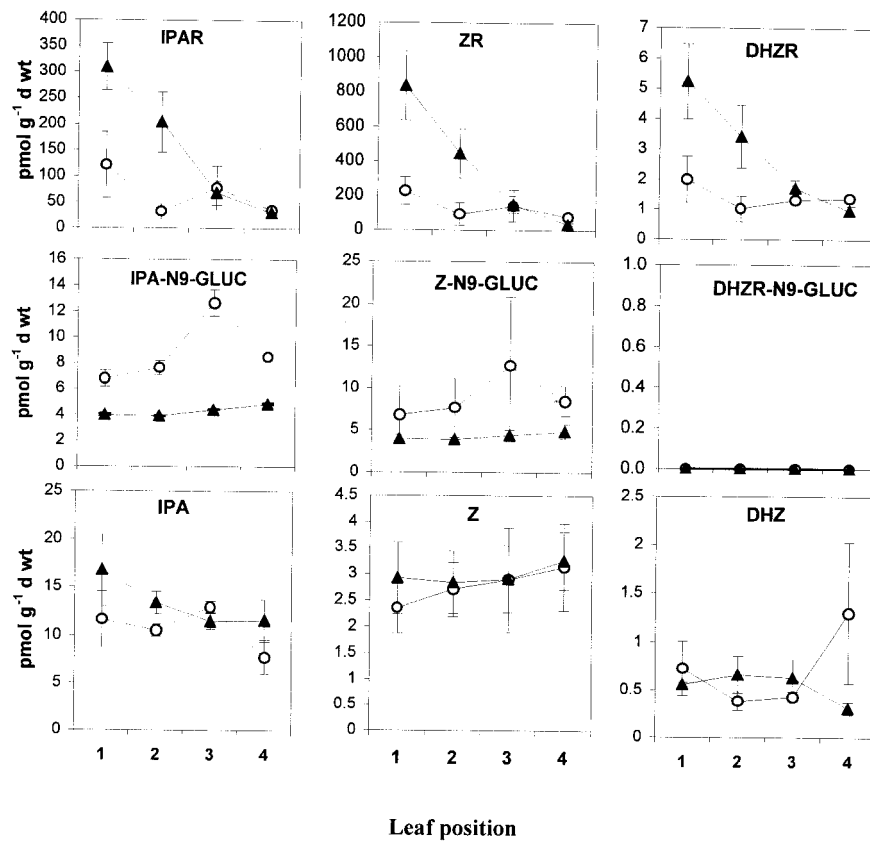
P_{SAG12} is repressed by sugars (Suc, Glc, and Fru), auxin (indole acetic acid), and cytokinins such as kinetin and benzyl adenine, but not by adenine (Noh and Amasino, 1999). The abnormally high concentrations of cytokinin and hexoses in the upper parts of P_{SAG12}-*IPT* lettuce were accompanied by poor flowering, "stay-green" panicles, and premature senescence of the upper leaves. Other research groups that have generated P_{SAG12}-*IPT* lettuce independently have observed the same phenotypic effects (K. Reinink, personal communication). These effects correlated with decreased concentrations of Rubisco, chlorophyll, and total soluble protein, suggesting that in the upper leaves of lettuce, photosynthesis was repressed by hexose accumulation. Although hexoses have been shown to repress P_{SAG12} activity, above a certain threshold these sugars also repress photosythesis and induce senescence; the latter would activate P_{SAG12}. As a consequence, instead of a negative feedback loop, positive feedback may occur in the upper parts of P_{SAG12}-*IPT* lettuce plants.

Autoregulation of P_{SAG12}-*IPT* during senescence may also depend on the availability of sufficient amounts of nutrients in the leaf. This seems to not be a problem in the outer leaves of vegetatively grown lettuce plants at 60 dps because P_{SAG12}-*IPT* is fully autoregulated. In developing upper leaves of prebolted plants (96 dps), nutrient supply via the root system probably terminated. As a consequence, these leaves depended on reallocation of nutrients from older, fully developed leaves. However, this route may be blocked in P_{SAG12}-*IPT* plants because proteins and chlorophyll were retained in older leaves (Fig. 3). Thus, senescence in upper leaves at 96 dps may well be induced by a lack of nutrients; P_{SAG12}-*IPT* is switched on, but is not autoregulated because of the shortage of nutrients. High concentrations of hexoses can be explained by the inverse relationship between nitrate and hexoses as osmoticum in plants (Blom-Zandstra and Lampe, 1985; Pritchard et al., 1996; McCall and Willumsen, 1999). If nitrate concentrations are low due to a block of reallocation and uptake via the root system, this can be compensated by increasing concentrations of hexoses.

The correlation between increased hexose accumulation and cytokinin levels in P_{SAG12}-*IPT* lettuce may provide insight into the mechanism by which cytokinin controls assimilate partitioning and, perhaps, plant development. It is well known that cytokinins increase the internal production of reducing sugars (mainly hexose and Fru) during cotyledon expansion (Huff and Ross, 1975). Accumulation of these sugars results in increased osmotic pressure, increased water uptake, and cell expansion (Bewli and Whitam, 1976), which probably accounts for the stem thickening and increased stem water content observed in P_{SAG12}-*IPT* lettuce.

The adverse effects of increased cytokinin and hexose levels, resulting from P$_{SAG12}$-IPT expression, on the reproductive stages of lettuce development demonstrates that during normal sequential senescence there is a highly coordinated balance between concentrations of cytokinin and sugars. The cytokinin-induced hexose accumulation has to be overcome to delay senescence. Wingler et al. (1998) proposed that to produce functional "stay-green" leaves, modification would be required of several regulatory mechanisms. Because cytokinins probably affect the transcription of a number of genes, it may be possible to identify, and to modify, the expression of those genes, thereby avoiding the pleiotropic effects associated with ipt expression. Studying the difference between expression of possible candidate genes in P$_{SAG12}$-IPT and non-transgenic plants may reveal alternative pathways for genetic manipulation to achieve an efficient delayed senescence strategy.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The binary vector pVDH394, based on the pBIN19 derivative pMOG18 (Sijmons et al., 1990), was introduced into *Agrobacterium tumefaciens* LBA4404 (Ooms et al., 1981) by triparental mating to give strain HAT1394. The T-DNA cassette in pVDH394 carried a 35S.nptII.35S gene next to the left border, a P$_{SAG12}$-IPT gene, and a 35S.gus-intron.35S gene adjacent to the right border (Fig. 10).

Bacteria were grown from −70°C glycerol stocks at 28°C on Luria broth (Sambrook et al., 1989) semi-solidified with 1.5% (w/v) agar and supplemented with kanamycin sulfate (100 mg L^{-1}) and rifampicin (50 mg L^{-1}). Overnight liquid cultures were incubated at 28°C on a horizontal rotary shaker (180 rpm) and were initiated by inoculating 20-mL aliquots of liquid Luria broth containing kanamycin sulfate (50 mg L^{-1}) and rifampicin (40 mg L^{-1}) in 100-cm^3 conical flasks. Cultures were grown to an optical density at 600 nm of 1.0 to 1.5 prior to inoculation of explants.
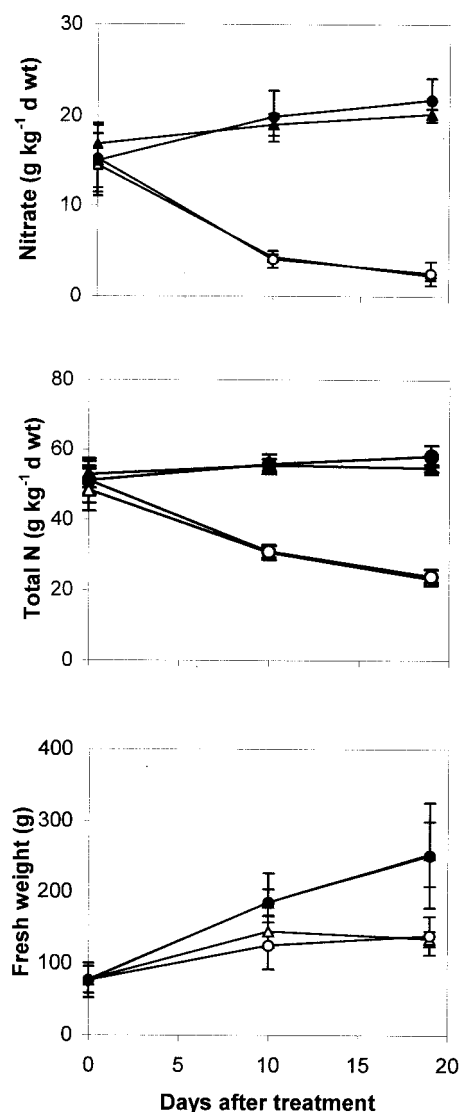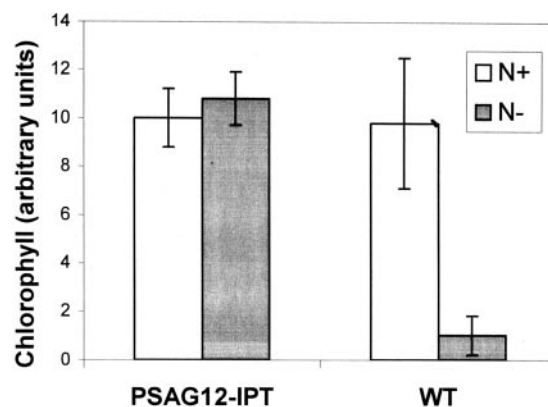
Figure 8. Total nitrogen, total nitrate, and fresh weight of heads of homozygous P$_{SAG12}$-IPT (1394.1.2; ▲) and corresponding azygous (●) plants grown in hydroponic culture (with nitrogen) and P$_{SAG12}$-IPT (△) and azygous (○) plants following removal of nitrogen from the medium at 53 dps (*n* = 3). Error bars represent SD.

In contrast to lettuce, the distribution of hexose sugars was reversed in both P$_{SAG12}$-IPT and non-transformed tobacco plants, with greater concentrations in the bottom leaves but lower in the upper leaves during flowering (Wingler et al., 1998). There are several possible reasons for these differences. For example, altering cytokinin levels is likely to have pleiotropic effects on overall gene expression (Chen et al., 1993) within the plant. Therefore, it is possible that increased cytokinin concentrations induce or suppress a different set of genes in lettuce compared with tobacco. Morphological differences between lettuce and tobacco may also contribute, such as leaf canopy density and heart formation that decrease light availability to the young leaves of lettuce.

Figure 9. Chlorophyll content of basal leaves of homozygous (1394.1.2) P$_{SAG12}$-IPT and corresponding azygous plants at 69 dps. N$^+$, Grown in hydroponic culture with nitrogen; N$^-$, nitrogen removed from medium at 53 dps.
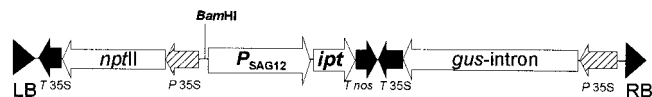
Figure 10. pVDH394 T-DNA. *P35S*, CaMV35S promoter; *T35S*, CaMV35S terminator; *Tnos*, nopaline synthase terminator; LB/RB, left and right T-DNA border sequences.

Plant Transformation

Lettuce (*Lactuca sativa* L. cv Evola) seeds were supplied by Leen de Mos ('s-Gravenzande, The Netherlands). Seeds were surface sterilized in 0.5% (w/v) sodium hypochlorite for 30 min, washed (three changes) in sterile distilled water, and placed on agar-solidified (0.8%, w/v) one-half-strength Murashige and Skoog (1962)-based medium with 1.0% (w/v) Suc, at pH 5.8 (20-mL aliquot/9-cm petri dish; 30–40 seeds/dish). Seeds were germinated and maintained at $23 \pm 2°C$ (16-h photoperiod, 350 μmol m^{-2} s^{-1}, daylight fluorescent tubes).

Cotyledons and the first true leaves from 7-d-old seedlings were inoculated with *A. tumefaciens* and transgenic shoots regenerated using an established procedure (Curtis et al., 1994). Shoots that regenerated from explants on medium containing kanamycin sulfate (50 mg L^{-1}) were rooted in vitro in the presence of kanamycin sulfate (50 mg L^{-1}) before transfer to the greenhouse, where they were allowed to self-pollinate and to set seed. Seeds were collected and stored at 4°C.

Reporter Gene Assays

GUS activity was determined histochemically and fluorometrically (Jefferson et al., 1987). Neomycin phosphotransferase II protein was detected according to the manufacturer's instructions using a DAS NPTII ELISA kit (5 Prime 3 Prime Inc., Boulder, CO).

Southern-Blot Analysis

Genomic DNA was extracted from young leaves using the method of Dellaporta et al. (1983). DNA was digested with *Bam*HI to generate T-DNA/plant DNA junction fragments. Following electrophoresis on 0.8% (w/v) agarose gels, digested DNA was transferred to nylon filters by alkaline capillary blotting and hybridized with a PCR-digoxigenin-labeled *ipt* probe. Bands that hybridized with the probe were visualized by non-radioactive chemiluminesce (McCabe et al., 1997).

Cytokinin Analysis

Purification, separation, and quantification of cytokinins were performed as described (Vonk et al., 1986; Jordi et al., 2000).

Analysis of in Planta Senescence

For all experiments, either wild-type or azygous plants (as indicated in text) were used as controls. In azygous plants, the transgene was lost during Mendelian segregation. When azygous plants were used as controls, they

were compared only with plants from the transgenic line from which they were derived. Seeds were sown on the surface of moist, M3 compost (Fisons, Ipswich, UK) in 9-cm-diameter plastic pots. The latter were placed in an incubator and the seeds germinated in a growth room at 19°C with a 16-h photoperiod (daylight fluorescent tubes; 350 μmol m^{-2} s^{-1}). At 7 dps, individual seedlings were transferred to 4- $\times$ 4- $\times$ 5-cm peat blocks and maintained under the same conditions. Individual plants were transferred at 30 dps to 9-cm plastic pots containing a mixture of John Innes No. 3 compost (J. Bentley, Barrow-on-Humber, UK):M3 compost Perlite (Silvaperl Ltd., Gainsborough, UK; 3:3:1 by volume). The pots were placed individually in 12-cm-diameter plastic trays each containing 5 to 10 mm of tap water, which was replaced every 24 h. Pots were spaced to 10 cm.

Induction of Senescence by Nitrogen Starvation

Two experiments were conducted. Seeds were germinated and were transferred 7 dps to hydroponic culture with a circulating nutrient solution (Steiner, 1984) containing nitrogen (N$^+$). The plants (30 azygous segregants and 30 second seed generation [T$_2$] homozygous GUS-positive P$_{SAG12}$-*IPT* plants) were grown in a greenhouse under natural daylight with 70% RH at 18°C day maximum/15°C night minimum temperatures. In experiment 1, natural daylight was supplemented with 276 μmol m^{-2} s^{-1} of daylight fluorescent illumination. In experiment 2, without supplementary lighting, the greenhouse was shaded when the light intensity exceeded 2,070 μmol m^{-2} s^{-1} to prevent tip burn. At 53 dps, the growth medium for 15 azygous and 15 P$_{SAG12}$-*IPT* plants was changed to nutrient solution lacking nitrate (N$^-$) prepared according to Jordi et al. (2000). For the N$^+$ treatment, the medium was replaced with new N$^+$ solution at 53 dps.

Analysis of Nitrate and Nitrogen Content

Total nitrogen and nitrate in dried plant material were determined by CHN analysis using a Heraeus carbon, hydrogen, nitrogen-rapid (Hanau, Germany) and a TRAACS800 continuous flow system (Bran and Luebbe Analyzing Technologies, Brixworth, UK), respectively (Bouma et al., 1994).

Postharvest Storage

Plants grown hydroponically were removed from N$^+$ and N$^-$ treatments at 61 and 69 dps and stored in boxes covered in transparent plastic film. A visual score was made of leaf yellowing.

Quantification of Chlorophyll

Using a plastic homogenizer cooled in liquid nitrogen, samples each of three frozen leaf discs (each 8-mm diameter) were crushed to a fine powder in 1.5-mL microfuge tubes followed by the addition of 1.2-mL aliquots of 80% (v/v) acetone. Samples were vortexed for 15 s and incubated in the dark at room temperature for 30 min with

inversion every 10 min. Bleached leaf material was removed by centrifugation (5 min; 10,000g) and 1-mL aliquots of supernatants transferred to new tubes. Chlorophyll a + b content of extracts was determined spectrophotometrically (Lichtenthaler, 1987). Nondestructive measurements were also made using a hand-held automated chlorophyll meter (SPAD-502; Minolta, Japan).

Quantification of Soluble Protein

Three frozen leaf discs were crushed to a fine powder in a microfuge tube; 100 μL of protein extraction buffer (60 mM Tris/HCl [pH 8.0], 500 mM NaCl, 10 mM EDTA, 30 mM β-mercaptoethanol, and 0.1 mM phenyl methyl sulfonyl fluoride) was added. Samples were vortexed for 15 s and insoluble leaf material removed by centrifugation (5 min; 10,000g). Supernatants were transferred to new tubes and stored at −80°C. Proteins were quantified spectrophotometrically (Bradford, 1976).

Quantification of LSU Rubisco

Rubisco was quantified by densitometry analysis of SDS-PAGE gels using Scion Image for Windows (Scion Corporation, Frederick, MD). Aliquots (7.5 μL) of protein extracts were electrophoresed using 12% (w/v) acrylamide gels before staining with Coomassie brilliant blue (RD250). The resulting LSU Rubisco bands were calibrated against the carbonic anhydrase bands of serial dilutions of protein standards (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

Quantification of Sugars

Sugars were extracted by homogenization of three leaf discs in 1 mL of buffered ethanol (100 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid],pH 7.5, 5 mM MgCl$_2$, and 90% [v/v] ethanol). Samples were incubated at 70°C for 10 min, centrifuged (5 min; 3,000g), and the supernatants retained. This was repeated three times for each sample. The supernatants from each sample were combined and dried at 50°C overnight. Dried extracts were resuspended in 1-mL aliquots of distilled water and stored at −20°C. Glc, Fru, and Suc were measured sequentially (Scholes et al., 1994).

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